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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Rudland et al.

Serial No. : 09/173,821

Filed : October 6, 1998

For : CONDITIONALLY IMMORTALISED CELL LINES DERIVED FROM TRANSGENIC ANIMALS

CLAIM FOR PRIORITY UNDER 35 U.S.C. § 119

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

A claim for priority is hereby made under the provisions of 35 U.S.C. § 119 for the above-identified continuation application of International Application PCT/GB97/01063 filed April 17, 1997, claiming priority of United Kingdom Application No. 9607953.8 filed April 17, 1998. These applications are listed in the declaration to the application, Serial No. 09/173,821, filed April 29, 1999. A certified copy of the United Kingdom application is enclosed.

Respectfully submitted,

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TRANSGENIC RAT

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TRANSGENIC RAT

The invention provides a transgenic rat which harbours a tissue-specific promoter which drives or controls expression of a cancer-causing gene or a cell-cycle effecting gene such as ts SV40T, c-Erb-B2 or TGFα. The presence of the promoter may predispose the transgenic rat to cancer, such as breast cancer, or to a cell proliferation disorder, for example by over-expression of the gene.

The invention further provides a cell-line derivable from the above transgenic rats.

The invention further provides transgenic tissue from the transgenic rat for experimental transplantation.

The invention further provides use of the transgenic rat or cell line or transgenic tissue for *in vivo* or *in vitro* identification or validation of drug targets and for the screening of drugs or candidate drugs. The transgenic rat may be used as an *in vivo* breast tumour model system. Immortalised neuronal cell-lines may be used as an *in vitro* system.

SUMMARY

Although transgenic mouse models for breast cancer have frequently been reported in the literature, transgenic rat models have not been described. We have generated transgenic rats overexpressing the human TGFα and c-*erb*-B2 genes in the mammary gland under the control of the mouse mammary tumour virus long terminal repeat promoter (MMTV-LTR), and have analysed multiple lines of these rats to the second (F_2) generation. Female MMTV-TGFα rats frequently develop severe hyperplasias during pregnancy, and a variety of tumours of long latency. The mammary glands of MMTV-αTGF rats fail to involute fully after the completion of lactation. Expression of the TGFα transgene is highest in the hyperplasias. MMTV-c-*erb*-B2 female rats develop a spectrum of benign and malignant lesions including ductal carcinoma *in situ* and carcinomas. Expression of the c-*erb*-B2 transgene is found in benign tumours such as fibroadenomas but is highest in the carcinomas. These animals model a spectrum of lesions found in human breasts and suggest that TGFα overexpression can act at a relatively early stage in the pathogenesis of breast cancer in the rat resulting in a predominantly hyperplastic response, whereas overexpression of c-*erb*-B2 plays a role in the induction of various benign lesions and more advanced breast carcinomas.

The Reason for Development of Transgenic Rat Models for Breast Cancer

The introduction of foreign genetic material into mice either by pronuclear microinjection into the germ line to create transgenic mice [1-3] or by transplantation of genetically modified mammary epithelium to create "transgenic tissue" [4] are powerful techniques which have contributed greatly to our understanding of the role that defined oncogenes play in the pathogenesis of breast cancer. However, the rat has certain potential advantages over the mouse for creating breast cancer models that accurately reflect the human disease. The origin, spectrum of tumour types in terms of their pathology, and hormone-sensitivity of spontaneously arising rat mammary tumours more closely resembles human breast cancer than that in the mouse (Table I) [5,6]. Unlike rat and human breast cancer, many mouse mammary tumours are viral in origin, caused by integration of mouse mammary tumour virus (MMTV). The common human benign breast tumours, such as the fibroadenoma, are also relatively common in ageing rats, but are virtually never seen in the mouse. Mouse mammary tumours are almost all epithelial and are either malignant from the outset or, if left long enough, become so. Moreover, mammary tumours in the rat are generally strongly hormone-dependent for both induction and growth. Carcinogen-induced rat mammary tumours regress after the same types of endocrine manipulations as do human cancers, and, as in the human disease, not all rat tumours show this hormone sensitivity. Rat mammary tumours induced by carcinogens or by implantation of estrogen pellets undergo regression after oophorectomy, adrenalectomy and hyperphysectomy, and, just as in the human disease, these effects are not permanent and the tumours eventually resume growth [5]. By contrast, most mouse tumours, virus-induced or otherwise, tend to be hormone insensitive, with the exception of the pregnancy-dependent mammary tumour described in BR6 mice [7].

Given the structural and functional resemblance of rat mammary

tumours to their human counterparts; it is not surprising that experimental induction of rat mammary tumours by methods such as administration of carcinogens or irradiation has been carried out for many years. In contrast to these relatively crude methods of induction, creation of transgenic rats that are predisposed to breast cancer by expressing defined oncogenes in the mammary gland enables a more defined analysis of the pathogenesis of the disease to be carried out at the molecular level.

There are two further reasons for creating transgenic rat models. Firstly, the results obtained from expressing a defined oncogene in the mouse may be peculiar to that species and may not be representative of its function in the human disease; it is therefore useful to establish whether expression of the gene has similar phenotypic effects in a relevant alternative species. Secondly, the rat is the preferred species for the screening of new pharmaceuticals and for toxicology/carcinogenicity testing [8]. Appropriate transgenic rat models may, therefore, be of some commercial value. Given that the rat mammary gland is sensitive to both chemical and radiation-induced carcinogenesis, rats predisposed to breast cancer may be particularly useful for carcinogenicity tests.

Choice of Oncogenes and Design of Constructs

In order to create transgenic rat models which mimic human breast cancer, we decided to target overexpression of the oncogenes TGFα and c-erb-B2 (HER-2) to the mammary glands.

TGFα is a mitogenic polypeptide that structually and functionally resembles Epidermal Growth Factor (EGF) [9]. TGFα is the most abundant member of the EGF family in the mammary gland, and it stimulates the growth of fibroblastic, myoepithelial-like and epithelial cells derived from normal mammary glands and benign tumours [10]. TGFα normally binds to

the EGF receptor (EGFR) and induces a mitogenic response by activating the tyrosine kinase activity of this receptor [11]. Immortalised mouse mammary epithelial cells have been shown to be susceptible to transformation by TGFα [12,13]. Moreover, overexpression of TGFα and its cognate receptor, EGFR, has been implicated in the pathogenesis of human breast cancers and it has also been shown that TGFα can mediate the growth-stimulating effects of estrogen in a human breast cancer cell line [17]. Minimal expression of inmmunoreactive TGFα is detectable in normal human breast tissue, but increased expression occurs in ductal hyperplasia, atypical hyperplasia and ductal carcinoma *in situ* [15]. Immunoreactive TGFα has also been detected in 30-70% of human breast carcinomas and its presence correlates with tumour burden [16-18].

Transgenic mice overexpressing TGFα from various promoters have been generated and characterised. MMTV-TGFα mice develop cystic and solid hyperplasias and mammary carcinomas in both multiparous and virgin animals after long latencies of >300 days [19,20]. These animals developed dramatically increased numbers of tumours of shorter latencies when treated with sub-optimal doses of the chemical carcinogen dimethylbezanthracene (DMBA) [21]. In one transgenic mouse model in which expression of TGFα was controlled by the metallothionein-1 promoter, secretory mammary adenocarcinomas and hyperplastic alveolar nodules developed [22], and in another model impeded morphogenic penetration of the ductal epithelial cells into the fat pad was reported even though DNA synthesis was enhanced in the transgenic mammary glands [23]. Although no mammary carcinomas developed in this latter model, the same construct induced hyperplasia, secretory mammary adenomas and adenocarcinomas in multiparous females of a different genetic background [24]. These animals also exhibited a delay in involution of the mammary tissue after lactation. These results suggested that, at least in the mouse, the susceptibility of the mammary gland to TGFα-

induced hyperplasia and neoplasia was specific to the strain used. Multiparous Whey Acidic Protein promotor driven-TGFα transgenic mice also developed well differentiated secretory adenocarcinomas with frequent induction of cyclin D1 expression, and also showed a delayed or inhibited involution of the mammary gland after lactation [25].

The c-*erb*-B2 gene product has also been strongly implicated in the development of human breast cancer. This proto-oncogene encodes a tyrosine kinase receptor that is structurally related to the EGF receptor [26,27] and is the human homologue of the transforming rat oncogene *neu* [28] which contains a point mutation in the transmembrane domain of the protein that results in constitutive tyrosine kinase activity [29].

The level of c-*erb*-B2 in normal human breast tissue is very low [30] but in invasive breast carcinomas, expression of c-*erb*-B2 is observed in 20-30% of breast tumours, which in some cases is accompanied by gene amplification [31-33]. An inverse correlation has been noted between patient survival and c-*erb*-B2 expression, particularly in patients with no involved lymph nodes [32,33]. In addition, almost 50% of early X-ray screened breast lesions of the ductal/lobular carcinoma-in-situ (cis) type express c-*erb*-B2 [34-36]. Its expression occurs in large cell, especially comedo-type *in situ* lesions, precursors for ductal carcinoma and often in pagets disease of the nipple [37].

Expression of *neu* in the mammary glands of certain lines of transgenic mice resulted in the rapid development of multifocal mammary tumours that metastasised at high frequency [38–40], but other laboratories have reported only the stochastic development of mammary tumours with MMTV/*neu* [41,42]. Perhaps surprisingly, expression of nonmutated c-*erb*-B2 in the mouse mammary gland did not induce development of tumours [42]. The reason for these discrepancies is unclear; perhaps the level of transgene expression or differences in the constructs are responsible.

In contrast, in a further MMTV-c-*erb*-B2 model, the animals died at an

early age as a result of hyperplastic lesions in both the kidneys and the lungs which caused organ failure. The mammary glands of these mice were underdeveloped and lactation deficient although one virgin mouse developed a focal adenocarcinoma [43]. Mammary tumours expressing the *neu* protooncogene have also been shown to possess elevated c-*src* yrosine kinase activity, suggesting that c-*src* is involved in *neu*-mediated signal transduction [44].

We decided to use the MMTV promotor linked to the Rous Sarcoma Virus (RSV)-LTR enhancer to drive expression of TGFα and nonmutated c-*erb*-B2 in the mammary glands. Constructs were made by subcloning human cDNAs for TGFα or c-*erb*-B2 downstream of the MMTV promotor. To provide an intron to enhance expression of the cDNAs and a splice and polyadenylation signal to ensure correct processing of the transcript, a 700bp fragment from the 3' end of the human growth hormone gene was placed downstream of the cDNAs (Figure 1). The completed transgenes were released from their parental plasmids by digestion with restriction enzymes, purified and microinjected into rat embryos.

Generation of Transgenic Rats

The generation of transgenic rats is similar, in theory, to the generation of transgenic mice. The only major difference is in the method of superovulation of the animals. Outbred Sprague Dawley female rats were superovulated by continuous fusion with pituitary Follicle Stimulating Hormone (FSH) via mini osmotic pumps [45]. Pumps were inserted intraperitoneally (i.p.) two days prior to mating. In contrast, the usual method for superovulating mice is to give a single injection of PMSG [46]. Synchronisation of ovulation was induced 48-52 hours later by i.p. injection of leutinising hormone releasing hormone (LHRH). Females were then mated

overnight with males of proven fertility. The following day, the females were sacrificed and embryos were collected in Dulbeccos PBS. When pronuclei were visible in the embryos, one pronucleus at a time (usually the male) was microinjected in modified M16 medium or M2 medium [46] . Embryos were then kept in modified M16 medium until they were transferred into the infundibulum of both oviducts of pseudopregnant recipients [47].

Rat embryos are far less resilient than those of the mouse; they are less elastic and spongier making them difficult to microinject without damaging them. Moreover, the pronuclei are more difficult to visualize than in mouse embryos. Furthermore, in our experience, oviduct transfers are less effective in the rat with fewer resulting pregnancies and generally smaller litter sizes.

Transgenic rats were identified by Southern blot analysis of tail genomic DNA, as for trangenic mice. The animals contained from less than one up to 50 copies of the integrated transgene per haploid genome. The characteristics of the founder transgenic rats are summerised in Table 2. With the exception of one of the male MMTV-TGFα founders, all the offspring were fertile and were mated successfully. Five of the founders transmitted the transgene in a Mendelian fashion to their offspring and four other founders transmitted the transgene at a much lower frequency; these latter founder animals were probably mosaics. No transmission was observed from one line of MMTV-TGFα transgenics and one of the MMTV-c-*erb*-B2 female animals appeared to be sub-fertile because only one litter was obtained; all the animals from this single litter were negative for the transgene. It was not possible to analyse any females from another of the MMTV c-*erb*-B2 lines (ERB /7) because the transgene appeared to integrate into the Y chromosome; all male offspring inherited the transgene but no female transgenic offspring were obtained from this animal (Table 2). All founders and multiple offspring from MMTV TGFα lines TGF/1 and TGF/2, and MMTV c-*erb*-B2 lines ERB/1 to ERB/ 3 have been analysed in detail to the second (F_2) generation. To date, we have monitored 29 female MMTV TGFα transgenics

and 34 female MMTV-c-*erb*-B2 transgenics for the development of mammary lesions that develop before 18 months of age. This analysis is ongoing, and a more complete analysis of the data discussed below will be reported in the future.

Mammary Lesions in MMTV-TGFα Transgenic Rats

MMTV-TGFα female transgenics were fertile and able to nurse their young normally. Virgin mammary epithelium showed no growth abnormalities and did not express the transgene at levels detectable by Northern blotting of poly(A)-RNA or by immunocytochemistry. The MMTV promotor is usually activated by the hormones of pregnancy, therefore rats were subjected to repeated rounds of pregnancy and lactation to activate expression of the transgene. The most striking phenotype observed was the development of large, solid palpable lumps in the mammary glands during pregnancy. These lumps appeared in 41% of transgenic female rats in both transgenic lines after five or more pregnancies. In the most severe cases lumps developed bilaterally in all the mammary glands. These lumps usually grew so large that the animals became moribund, necessitating culling. The lumps always appeared on day 10 or day 11 of pregnancy and invariably regressed the day before parturition, suggesting that they were severe hyperplasias rather than neoplasias. The animals were still able to lactate normally and nurse their young in the subsequent lactational period following regression of these lesions. However, the lumps usually reappeared with greater severity during subsequent pregnancies. When these lesions were examined histologically, they were found to consist of solid masses of tissue resembling normal lactating mammary gland. The hyperplastic mammary tissue compressed around normal tissue such as skeletal muscle but did not invade it. The hyperplastic mammary tissue

stained with antiserum to TGFα. Whole-mounts of mammary tissues from other pregnant transgenic female animals showed that the mammary tissue was always hyperplastic in comparison with non-transgenic litter mates of comparable age and number of pregnancies. In transgenic females the fat pad became completely filled with proliferating mammary epithelium and individual lobules were impossible to distinguish because they merged together ~~(Figure 2)~~.

The mammary glands of transgenic female animals also failed to regress fully after lactation; dense, focal hyperplastic lobules with secretions persisted in these animals, even six months after their previous lactation. These hyperplastic lesions also stained with TGFα antiserum. Involuted mammary glands from control litter mates after comparable numbers of pregnancy and lactation cycles were very different, consisting of small condensed ducts and alveoli with no evidence of lactation.

Tumours developed stochastically after a long latent period in multiparous females; by 18 months of age 8 out of 29 (28%) of animals had developed tumours. These tumours were variable histologically; and included fibromas, benign papillary tumours with associated severe hyperplasia, ductal carcinoma *in situ* (DCIS) and carcinomas with squamous metaplasia. Transgene expression was variable; in fibromas the fibroblastic cells that made up the majority of the tumour stained strongly, whereas in DCIS and carcinomas expression of TGFα was either absent or non-uniform. However, strong expression of TGFα was always seen in adjacent hyperplastic breast tissue, when present, and in carcinomas where differentiation to squamous elements occurred (Figure 2).

Mammary Lesions in MMTV-c-*erb*-B2 Transgenic Rats

MMTV c-*erb*-B2 transgenic females did not develop the severe pregnancy-responsive hyperplasias characteristic of the TGFα transgenics. Indeed, whole-mounted mammary glands of pregnant transgenic females did not reveal any evidence of hyperplasia. Transgene expression was not detectable in virgin females, and only just detectable in pregnant animals by immunocytochemistry and by Northern blotting of poly-(A)-containing RNA.. However, whole-mounted mammary glands from females at least six weeks after their previous lactation revealed focal areas of mild or moderate adenosis/hyperplasia (~~Figure 3~~). These mildly hyperplastic regions stained moderately or weakly on their plasma membrane with antiserum to c-*erb*-B2, (Fig 3a) whereas cells in regressed, condensed alveoli in the same mammary gland failed to stain with antiserum to c-*erb*-B2. Although not as pronounced as in the MMTV-TGFα transgenics, c-*erb*-B2 expression does appear to be correlated with retention of hyperplastic secretory alveoli.

Analysis of otherwise involuted mammary glands from multiparous transgenic females also revealed a variety of other pathologies. These included collections of thick ducts, which when sectioned appeared to be large cystic expansions, and multiple areas of focally dense tissue. When sectioned, these dense areas were usually found to be small fibroadenomas (Fig 3b) or other benign lesions including sclerosing adenosis (Fig 3c). These benign lesions are likely to be due to transgene expression and not arise spontaneously for three reasons. Firstly, they were multifocal. Secondly, they were observed very infrequently in mammary glands from control litter mates of comparable age and reproductive history. Out of 20 control females only one area of mild fibroadenomatous change was found and areas of hyperplasia were not observed. Thirdly, both cystic expansions and fibroadenomas (Fig 3) stained with antiserum to c-*erb*-B2, (Fig 3d) whereas surrounding normal mammary tissue failed to stain.

As for the MMTV-TGFα transgenics, tumours developed stochastically at low frequency after multiple pregnancies. These tumours included large

fibroadenomas and histologically variable tumours with a papillary growth pattern where the papillary epithelium lined cystic spaces in which dense secretions were present. Areas of DCIS were also present within these lesions suggesting that a progression occurs from hyperplasia to papillary lesions and then to DCIS. To date 3 animals have developed DCIS and only 2 animals have developed definite carcinomas. Although the carcinomas were well differentiated in comparison with most human breast carcinomas, they were poorly organized in comparison with the benign tumours and contained more malignant-looking cells with large, pleiomorphic nuclei. They were classified as definite carcinomas because they failed to stain with antisera to keratin and smooth muscle actin indicating the absence of myoepithelial cells; moreover staining with antiserum to laminin revealed that basement membrane was either absent or very fragmented. Although cells in areas of DCIS stained weakly with c-*erb*-B2 antiserum, cells in carcinomas stained very strongly indeed on their membranes, whilst adjacent normal mammary ductal epithelium failed to stain.

Other Nonmammary Lesions in Transgenic Rats

Although the MMTV-TGFα and MMTV-c-erb-B2 transgenes were also variably expressed in several other tissues, including the epithelial cells of the male reproductive tract, salivary glands and areas of the kidneys and spleen, no apparent pathologies were observed in these tissues, with the notable exception of the salivary glands, where hyperplasia sometimes occurred in both MMTV-TGFα and MMTV-c-*erb*-B2 transgenic rat lines. These results suggest that either these other tissues are not susceptible to TGFα- and c-*erb*-B2-induced carcinogenesis or that the level of transgene expression is not sufficient to induce a neoplastic phenotype in these tissues.

The only other striking phenotype of note was areas of hair loss, especially ventrally, in many of the MMTV-TGFα transgenics. Transverse sections through the skin revealed areas of sebaceous gland hyperplasia. These hyperplastic sebaceous glands stained intensely with antiserum to TGFα whereas the remainder of the dermis failed to stain and appeared to be normal. Dense hyperplastic mammary tissue was nearly always found in close proximity to these sebaceous gland hyperplasias, but it did not invade the sebaceous glands and hair follicles of the dermis. Therefore, sebaceous gland hyperplasia is most probably responsible for hair loss in these animals.

A Model for the role of TGFα and c-*erb*-B2 Overexpression in Rat Mammary Carcinogenesis

The results from our transgenic rat models suggest that overexpression of human cDNAs for TGFα and c-*erb*-B2 in the rat mammary gland can predispose such animals to tumour development. However, the spectrum of preneoplastic and benign lesions and pattern of transgene expression in the various lesions suggests that the two genes act at different stages in the pathogenesis of the disease.

Expression of TGFα appears to cause predominantly hyperplasia. Although a certain degree of mammary hyperplasia is always present in MMTV-TGFα transgenic rats even during the first pregnancy, the severe, macroscopically identifiable hyperplasias that resemble lactating adenomas only appeared after five or more pregnancies and then only in approximately half of the animals. This suggests that either a critical threshold level of transgene expression is needed for the severe hyperplasias or that a second, cooperating genetic event needs to take place for these hyperplasias to develop. In addition to the induction of secretory hyperplasias, the TGFα transgene also appears to prevent involution of the lactating mammary gland

by enhancing epithelial cell survival. Hyperplasia and failure of the mammary glands to involute fully are two phenotypes that have been described in certain lines of transgenic mice expressing TGFα in the mammary gland [19-25]. Sebaceous gland hyperplasia has also been observed in one line of MMTV-TGFα transgenic mice [20]. However, the severe, pregnancy-associated hyperplasias that are characteristic of our transgenic rats have not been described in TGFα transgenic mice, although pregnancy-dependent lesions similar to those seen in the BK6 mouse strain have been described in MMTV-int-2-expressing mice [48]. This may be because these lesions are hormone-dependent and lends further support to the rat as a model of hormone-responsive mammary tumour development. We are not sure at this stage whether these severe hyperplastic lesions can progress to hormone independence, because their severity has necessitated early culling of the animals; it will be necessary either to resect them partially or attempt to transplant them into oophorectomised or hypophysectomised recipient rats to prove this progression.

MMTV-TGFα transgenic rats develop tumours at low frequency, but expression of the transgene in the carcinomas is frequently low or absent, suggesting that further undetermined genetic events are responsible for the development of these more advanced lesions, and that TGFα expression is unnecessary. However, areas of hyperplasia and papillary lesions adjacent to and continuous with such advanced lesions stain intensely with antiserum to TGFα; this result suggests that the more advanced lesions can develop from these preneoplastic lesions which express the transgene. TGFα clearly can also stimulate proliferation of the stromal fibroblastic cells of the mammary gland; the large fibromas which develop in our MMTV-TGFα transgenic rats strongly express TGFα.

Whilst TGFα overexpression has been detected in both mammary epithelial and fibroblastic cells, c-*erb*-B2 overexpression has only been

detected in epithelial cells in the various lesions that developed in MMTV-c-*erb*-B2 transgenic rats. The c-*erb*-B2 gene is expressed in epithelial cells of fibroadenomas, mild hyperplasias and large cystic expansions induced in such animals but not in fibroblasts. Overexpression of c-*erb*-B2 appears to play a role in the induction of benign breast diseases and in benign tumours in these animals; fibroadenomas are particularly commonly found. However, expression of c-*erb*-B2 was strongest in the carcinomas that developed in these transgenics. Our MMTV c-*erb*-B2 transgenic rat model most closely resembles the transgenic mouse model of Bouchard *et al.* [41] in which tumours developed stochastically after a long latent period, although the occurence of benign lesions were not reported in this model. However, development of benign lesions such as adenosis, sclerosing adenosis and the preneoplastic lesion DCIS was reported when *neu* was expressd in the reconstituted mouse mammary gland [50].

Taken together these results suggest a model where TGFα acts at an early stage in breast cancer pathogenesis predominantly causing hyperplasia and retention of lactating alveoli, and c-*erb*-B2 induces certain benign breast lesions and is important in the development of carcinoma (Figure 5).

Role of Mammary differentiation in MMTV-Driven Transgenics: Possible Limitations on Malignant Development

The development of malignant carcinomas is infrequent in our MMTV-c-*erb*-B2 transgenic rats. One possible reason for this may lie in the choice of the MMTV promotor for creating the models. The MMTV-LTR promotor is known to be activated by hormones during pregnancy and lactation such as glucocorticoids and prolactin [48]. In our models, we have confirmed that transgene expression is absent in virgin mammary epithelium and induced during pregnancy. In humans over a whole lifetime, epidemiological

evidence has shown that pregnancy protects against the development of breast cancer. This is particularly true of pregnancy early in reproductive life [51,52]. It has been suggested that early first pregnancy protects the breast by causing some of the stem cell population to differentiate, thus removing the cells from the pool of dividing cells. Less cells are therefore available to mutate and form tumours. Late first pregnancy is not as protective because mutations accumulate over time in the stem cell population and the growth-inducing effects of pregnancy may then encourage expansion of the mutated cell population [52]. Moreover in the rat, completion of pregnancy and lactation before exposure to carcinogens such as DMBA markedly reduces the susceptibility of the mammary gland to chemical carcinogenesis [6]. Studies from carcinogen-induced rat mammary tumours have shown that malignant mammary carinomas mainly arise from undifferentiated terminal end buds (TEBs) of the gland. Administration of DMBA to virgin 45-55 day old rats during the period in which TEBs are decreasing in number due to their differentiation into alveolar buds (ABs) causes affected TEBs to develop large intraductal proliferations of epithelial and intermediate cells instead of differentiating into ABs. These proliferations become progressively larger and may eventually develop into DCIS and further if transplanted against an immunological barrier [53]. In contrast, those TEBs that have differentiated already into ABs before DMBA administration, do not develop DCIS but either remain unmodified, undergo dilation giving rise to hyperplastic lobules or cystic dilations, or exhibit ductular proliferation to form benign tumours such as adenomas or fibroadenomas [6]. The observation that mammary carcinomas probably arise from the undifferentiated stem cell-containing structures of the gland (TEBs), whereas benign tumours such as adenomas and fibroadenomas arise from structures that were more differentiated at the time of carcinogen administration, suggests that the more differentiated the structure at the time of carcinogenic insult or indeed oncogenic activation, the more benign and organised is the tumour that

develops. Similar results have been obtained when the neu oncogene is expressed in the reconstituted mouse mammary gland [54]. Moreover in our transgenic rats benign intraductal proliferations, intraductal papilloma and DCIS occur together suggesting progressive steps in a carcinogenic pathway.

Improvements in the Transgenic Model

In view of the above, carcinoma development may have been more frequent if the animals had remained virgins for a long period of time before their first pregnancy. During this period the additional mutations needed to co-operate with the c-*erb*-B2 gene to induce carcinoma development may occur; carcinomas may then develop during the first pregnancy/lactation period, when expression of the c-*erb*-B2 transgene is induced. Further studies will be necessary to test this hypothesis. Ideally it would be preferable to obtain a promotor active in a mammary stem cell population to target expression of the oncogene to rat mammary epithelial stem cells before differentaition occurs to ABs. However, to our knowledge, such a promotor has not yet been cloned and characterised.

Despite the limitations of the MMTV promotor, the transgenic rat models we have developed have provided some insight into their roles in development of rat mammary cancers. In future studies we hope to address the following questions. (i) Can the frequency of carcinoma development be increased by keeping the animals in a virgin state for a long period of time before first pregnancy? (ii) Can the TGFα and c-erb-B2 gene products cooperate in breast cancer development? (iii) Are the transgenic rats more sensitive to chemical carcinogenesis? (iv) Can the TGFα-induced hyperplasias progress to hormone-independent growth and are they are transplantable? Moreover it is hoped that we can culture cell lines from the various preneoplastic lesions and transfect them with further candidate oncogenes, antisense constructs to tumour suppressor genes and metastasis-associated genes to gain further insight into the multi step process of

carcinogenesis in the rat mammary gland.

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Table 1: Comparison of Pathology of Human, Rat and Mouse Mammary Tumours

	Man	Rat	Mouse
Benign: Maignant ratio	about 1:4	benign more common	usually malignant
Fibroadenoma	common	common	very uncommon
carcinoma:			
Spontaneous	common	uncommon	common
Induced	-	uncommon	common
Differentiation:			
Range	Wide	Wide	limited
Degree	Poorly Differentiated	Highly Differentiated	very highly differentiated
Hormone-response	Sometimes Positive	Sometimes Positive	not in virus induced tumours; only in BR6 strain

Modified from Young and Hallowes (1973)

Table 2: Generation of Transgenic Rats

MMTV-TGFα

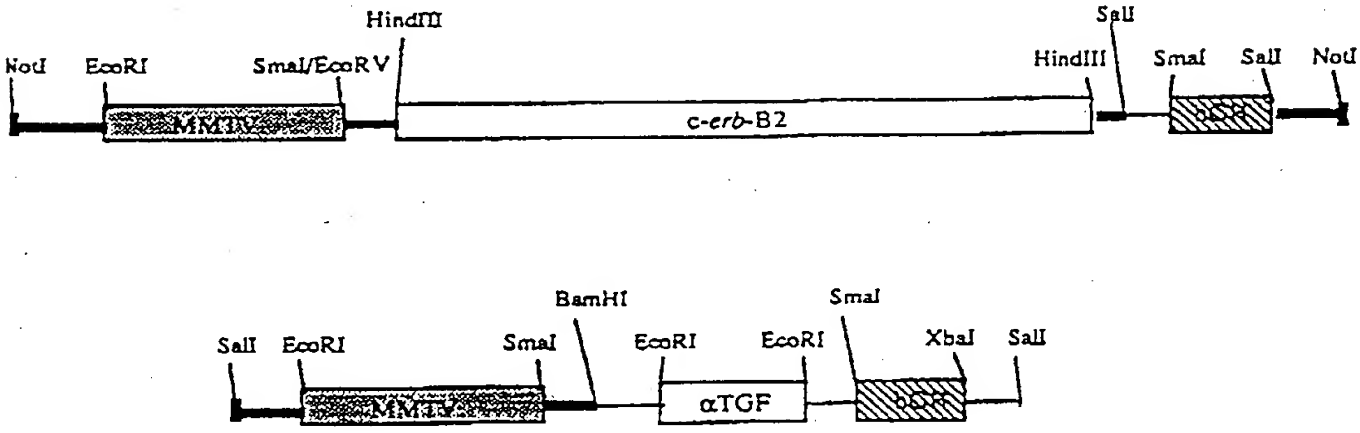
Founder	Sex	Copies of transgene	Successfully mated	Mosaic or Mendelian Inheritance
TGF/1	Female	5-10	Yes	Mendelian
TGF/2	Female	1	Yes	Mosaic
TGF/3	Male	10	Yes	Mosaic
TGF/4	Male	10	Yes	Mosaic
TGF/5	Male	5-10	Yes	No transmission
TGF/6	Male	1-5	No	Unknown

MMTV c-Erb-B2

ERB/1	Female	10-20	Yes	Mendelian
ERB/2	Female	1-5	Yes	Mendelian
ERB/3	Female	~ 50	Yes	Mendelian
ERB/4	Male	1 and > 20	Yes	Mendelian Two Integration Sites
ERB/5	Female	< 1	Yes	Unknown
ERB/6	Female	5-10	Yes	Mosaic
ERB/7	Male	1	Yes	Sex linked - transgene on Y chromosome

FIG 1

Figure 1: Structure of the MMTV-TGFα and MMTV-c-Erb-B2 transgenes
The diagram shows important restriction sites which were utilised in the construction of the transgenes.



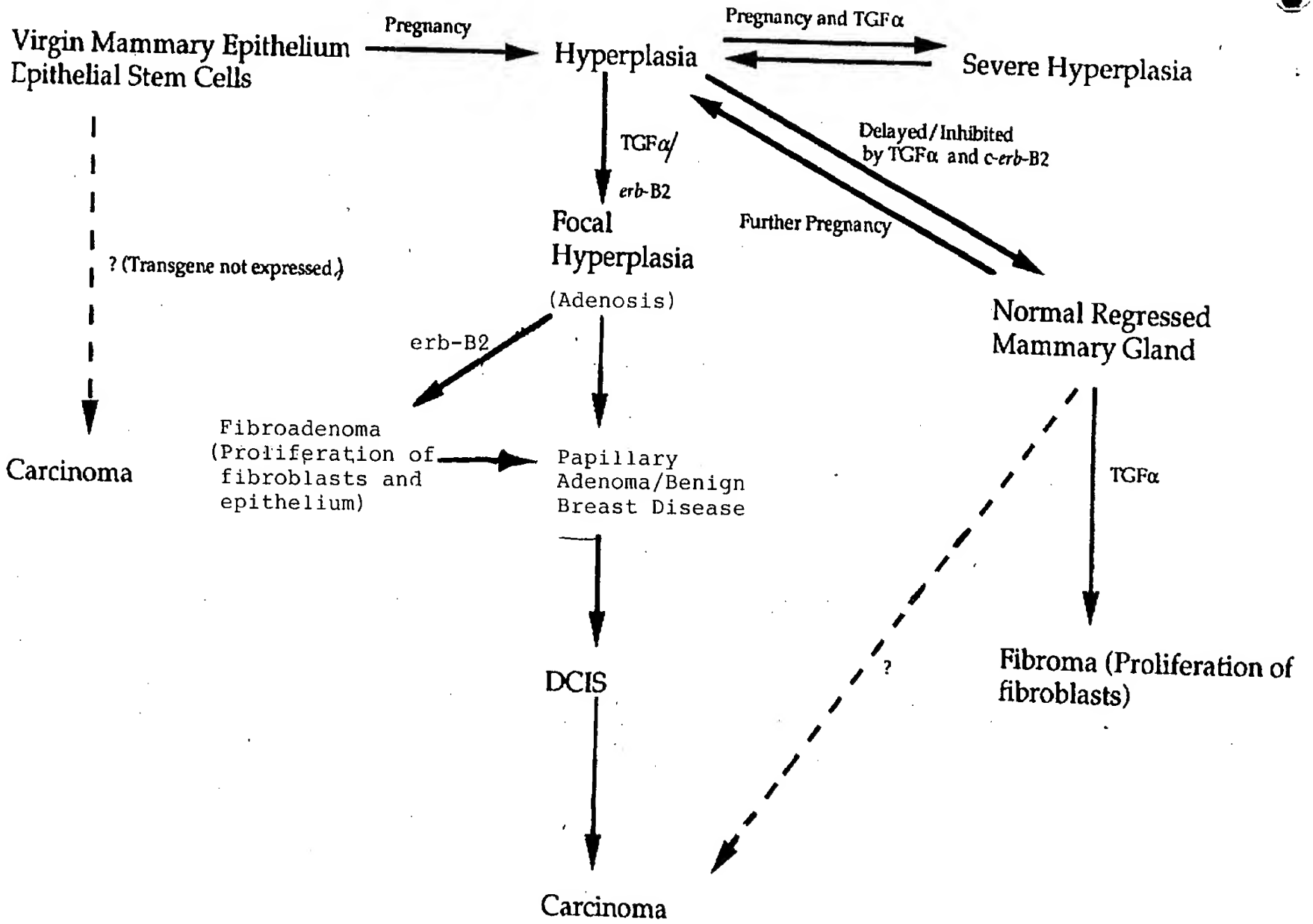

Virgin Mammary Epithelium
Epithelial Stem Cells
Pregnancy
Hyperplasia
Pregnancy and TGFα
Severe Hyperplasia
TGFα/
erb-B2
Delayed/Inhibited
by TGFα and c-erb-B2
Further Pregnancy
Focal
Hyperplasia
(Adenosis)
? (Transgene not expressed)
erb-B2
Normal Regressed
Mammary Gland
Fibroadenoma
(Proliferation of
fibroblasts and
epithelium)
Papillary
Adenoma/Benign
Breast Disease
Carcinoma
TGFα
DCIS
?
Fibroma (Proliferation of
fibroblasts)
Carcinoma

FIG 4

2/2